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Nerfin-1 is required for early axon guidance decisions in the developing *Drosophila* CNS

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Abstract

Many studies have focused on the mechanisms of axon guidance; however, little is known about the transcriptional control of the navigational components that carryout these decisions. This report describes the functional analysis of Nerfin-1, a nuclear regulator of axon guidance required for a subset of early pathfinding events in the developing *Drosophila* CNS. Nerfin-1 belongs to a highly conserved subfamily of Zn-finger proteins with cognates identified in nematodes and man. We show that the neural precursor gene *prospero* is essential for *nerfin-1* expression. Unlike *nerfin-1* mRNA, which is expressed in many neural precursor cells, the encoded Nerfin-1 protein is only detected in the nuclei of neuronal precursors that will divide just once and then transiently in their nascent neurons. Although *nerfin-1* null embryos have no discernible alterations in neural lineage development nor in neuronal or glial identities, CNS pioneering neurons require *nerfin-1* function for early axon guidance decisions. Furthermore, *nerfin-1* is required for the proper development of commissural and connective axon fascicles. Our studies also show that Nerfin-1 is essential for the proper expression of *robo2*, *wnt5*, *derailed*, *G-oα47A*, *Lar*, and *futsch*, genes whose encoded proteins participate in these early navigational events.
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Keywords: Nerfin-1; EIN domain Zn-finger proteins; Axon guidance; CNS development

Introduction

During their journey to synaptic targets, axon growth cones respond to multiple guidance cues, both attractive and repulsive; the components of these navigational events include secreted ligands, transmembrane receptors, signaling proteins, and the transcriptional/translational factors that regulate their expression (reviewed by Huber et al., 2003; Tessier-Lavigne and Goodman, 1996). Pioneering axons depend on both signaling cues from and physical contact with glia as they project through different molecular environments (Hidalgo and Booth, 2000).

Early pathfinding decisions in the developing *Drosophila* ventral cord are governed in part by at least three distinct receptor–ligand signaling events (reviewed by Huber et al., 2003; Schnorrer and Dickson, 2004; Yoshikawa and Thomas, 2004): (1) axonal attraction to the midline is regulated by Netrin/Frazzled signaling (Harris et al., 1996; Mitchell et al., 1996); (2) the repulsive ligand Slit, acting in conjunction with multiple Robo receptors, mediates midline crossing and choice of alternative longitudinal fascicles (Kidd et al., 1999; Rajagopalan et al., 2000; Simpson et al., 2000a,b); and (3) the Wnt5 ligand and its receptor Derailed help regulate the decision to project through either an anterior commissure (AC) or posterior commissure (PC) (Fradkin et al., 2004; Yoshikawa et al., 2003).

Although much is known about the molecular details of receptor–ligand interactions and subsequent second-messenger signaling events that occur at the growth cone, less is understood about the transcriptional regulation of the genes

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that encode the navigational machinery. The cell-type-specific and temporal expression dynamics of axon guidance determinants in the developing nervous system suggests that their encoding genes are under tight transcriptional regulation. Mutant analysis of a number of vertebrate transcription factors involved in neural fate specification has shown that these regulators are also required for specific axon guidance events; for example, disruption of the murine Pax6 (Jones et al., 2002), Brn-3 (Erkman et al., 2000), Nkx6.1 (Muller et al., 2003), and LIM homeodomain transcription factors (Kania and Jessell, 2003) each results in axon pathfinding errors. Likewise, in *Drosophila*, a number of multifunctional transcriptional factors are required for proper axon guidance: These include *even-skipped* (*eve*; Fujioka et al., 2003), *fruitless* (*fru*; Song et al., 2002), *longitudinals lacking* (*lola*; Giniger et al., 1994), and *prospero* (*pros*; Doe et al., 1991; Vaessin et al., 1991).

This report describes the functional analysis of *nerfin-1*, a novel axon guidance gene that relies on *pros* for its full expression. Initially discovered in a screen for neural precursor genes, *nerfin-1* encodes a member of a subfamily of Zn-finger proteins with cognates identified in all metazoans (Brody et al., 2002; Stivers et al., 2000). Nerfin-1 and other family members contain a highly conserved set of tandem Zn-fingers termed the EIN domain [named after the first three identified members: nematode *egg laying-46* mutant (*egl-46*) (Desai and Horvitz, 1989; Desai et al., 1988); human insulinoma associated-1, IA-1 (Goto et al., 1992); and *Drosophila nerfin-1* (Stivers et al., 2000)]. Loss of *egl-46* function causes multiple defects in *C. elegans* nervous system development including abnormalities in cell migration, axonal outgrowth, and neurotransmitter production (Wu et al., 2001; Yu et al., 2003). The mammalian IA-1, shown to be a sequence-specific DNA binding protein (Breslin et al., 2002), is expressed during CNS development and in neuroendocrine tumors (Breslin et al., 2003 and references therein).

Although *nerfin-1*^{null} mutant embryos have no discernible alteration in either CNS or PNS lineage development, loss of *nerfin-1* triggers axonal patterning defects throughout the CNS, but not in the PNS. Our studies demonstrate that Nerfin-1 is required for the proper expression of a subset of factors that participate in early axon guidance decisions essential to both commissural and longitudinal connective axon fascicle development. Analysis of *nerfin-1* expression reveals that Nerfin-1 protein is detected in only a subset of neural precursor cells that express its encoding message. Nerfin-1 accumulates in the nuclei of only those precursor cells that divide just once producing neurons and then transiently expressed in their nascent offspring. Prolonged expression of Nerfin-1 in neurons interferes with later stages of CNS axon fasciculation and patterning. Finally, we show that *nerfin-1* is downstream of *pros* in the regulatory network(s) controlling axon guidance determinants. Epistasis experiments also demonstrate that the regulatory networks controlling *nerfin-1* expression and those regulating the expression of *lola* or *fru* are separate from one another.

Materials and methods

Drosophila P-element transformants and stocks

Germ-line transformants were generated using standard techniques by coinjecting *Df(1)w*^{67c}*,y* embryos with constructs, described in Supplemental materials, and the helper plasmid p 25.7wc (Spradling, 1986). Mutant alleles of *pros*, *fru*, *lola*, and the GFP expressing pCC/aCC/RP2 line are also described in the Supplemental materials. Standard animal husbandry procedures were used in the care and handling of *Drosophila* stocks (Ashburner, 1989).

Generation and molecular characterization of nerfin-1 deficiencies

For the targeted disruption of the *nerfin-1* locus, the ends-in homologous recombination knockout technique of Rong and Golic (2001) was used. Information about the knockout construct, including cloning steps, is available in the Supplemental materials. Additional deficiencies covering the *nerfin-1* locus were generated by di-epoxybutane or X-ray mutagenesis of the initial targeted insertion line marked by w^+ (mutagenesis protocols are described in Ashburner, 1989). Details are available upon request.

nerfin-1 rescue and misexpression experiments

Two independent 2nd chromosome insertions of a Casper3 P vector/genomic DNA rescue construct were used to rescue the *Df(3L)nerfin-1*^{54} allele. Full details of the rescue construct are available upon request. The PCR derived 11,154 bp genomic rescue DNA fragment contained the *nerfin-1* transcribed sequence plus 5780 bp of 5′ and 2130 bp of 3′ flanking sequence. Misexpression of Nerfin-1 was accomplished using the *GAL4-UAS* strategy (Fischer et al., 1988). After insertion of the 1422 bp *nerfin-1* open reading frame into the P-element vector (pUAST, Brand and Perrimon, 1993), the orientation and reading frame was confirmed by DNA sequence analysis. Males of the P[*UAS.nerfin-1*] 33-1 line (homozygous for insertions on the 2nd and 3rd chromosomes) were crossed with virgin females of the different *GAL4* driver lines to ectopically express Nerfin-1 (see Supplemental materials for *Gal4* lines). For each *Gal4 driver/UAS.nerfin-1* combination, Nerfin-1 misexpression was confirmed by anti-Nerfin-1 immunostaining.

Antibody production, immunohistochemistry, and mRNA localization

Histidine-tagged fusion proteins corresponding either to the first 207 N-terminal or the last 169 C-terminal amino acids of the predicted Nerfin-1 protein were expressed in bacteria by the pET expression system (Studier et al., 1990) and purified according to the manufacturer's protocol

(Novagen). Recombinant proteins were used to immunize both rabbits and guinea pigs (details available upon request). Embryo fixation and whole-mount immunostaining were carried out according to the procedures described in Patel (1994). Primary antibodies, their final dilutions, and source references are listed in Table 1. Rabbit anti-GFP was obtained from Invitrogen. Vectastain ABC second antibody avidin/biotin HRP visualization reagents were used according to the manufacturer's protocol (Vector Labs). For in situ hybridization, single-strand riboprobes were prepared as previously described (Kopczynski et al., 1996), with the exception that riboprobes were prepared from PCR-amplified cDNAs with a labeling mix containing Fluorescein-12-UTP (Roche) and visualized using anti-FITC Fab fragments coupled to alkaline phosphatase. cDNAs were obtained from the BDGP (listed in Table 1) and detailed protocols are available upon request. Embryos and dissected fillets were viewed in 70% glycerol/phosphate-buffered saline (PBS) and photographed using a Nikon Optiphot microscope equipped with DIC/Nomarski optics. Embryo developmental staging was determined by morphological criteria (Campos-Ortega and Hartenstein, 1985). For confocal microscopy, FITC-, Cy3-, or Cy5-conjugated secondary antibodies (Jackson Labs) were used. Images were collected on a Zeiss LSM 410 equipped with a krypton/argon laser.

Results

Nerfin-1 protein is detected in a subset of neural precursor cells and neurons that express nerfin-1 mRNA

Nerfin-1 protein expression was studied using polyclonal antibodies raised against unique N- and C-terminal regions of the predicted 469 amino acid protein. The specificity of each antiserum was confirmed by the absence of Nerfin-1 immunostaining in embryos homozygous for *nerfin-1*^{null} mutations (see below). During embryonic stages 7 through 9, Nerfin-1 encoding transcripts were found in all early delaminating ventral cord NBs, albeit at differing levels (Fig. 1A inset; Stivers et al., 2000). In marked contrast, immunostaining with both Nerfin-1-specific antisera identified only four ventral cord NBs per segment that expressed Nerfin-1 protein (Fig. 1A–C). These NBs, the unpaired midline MP1 and MP3 and the lateral MP2 pair, are unique: Unlike other ventral cord NBs, they do not undergo multiple asymmetric GMC producing divisions during CNS development but rather divide just once to generate interneurons (Schmid et al., 1999). The identity of the MP2 NB as the sole Nerfin-1-positive lateral NB was established by first determining that one of its medial row NB neighbors, on its posterior flank, was the 5–2 NB (see Supplemental materials for data; for NB map, see Broadus et al., 1995); this identification was subsequently confirmed by co-nuclear localization of Nerfin-1 and the Prospero (Pros) homeodomain protein (see arrowhead in Fig. 1B inset); except for its nuclear localization in the MP2, Pros is excluded from the nucleus in all other ventral cord lateral NBs. Following the MP2 NB division, Nerfin-1 was detected in both the vMP and dMP nascent interneurons (Fig. 1C, arrowhead). Shortly after the onset of Nerfin-1 expression in the MP2 NBs, the unpaired midline MP1 and MP3 NBs and their nascent neurons also transiently express Nerfin-1 (Figs. 1B, C).

By stage 12, *nerfin-1* mRNA expression is activated in most newly formed CNS GMCs and nascent neurons (Fig. 1C, inset; Stivers et al., 2000). This appears to be de novo activation of gene expression, since at stage 11, *nerfin-1* mRNA is absent from NBs (Stivers et al., 2000). Nerfin-1 and Prospero protein co-localization studies revealed that many, but not all, Prospero-positive cells express Nerfin-1 (Fig. 1E; see also Figs. 2 and 7 for Nerfin-1 expression). By early stage 13, many newborn neurons during the initial phase of their axon development express Nerfin-1, as judged by double immunolabeling with anti-Nerfin-1 and the neuron-specific anti-Elav antibody (data not shown). However, both *nerfin-1* mRNA and protein expression in neurons is transient. Starting at late stage 13, there is a progressive reduction in the number of neurons that express *nerfin-1* mRNA or protein such that by late stage 14, only a small subset of cells throughout the CNS has detectable levels of expression (Stivers et al., 2000).

In the developing PNS chordotonal and external sensory (ES) organs, Nerfin-1 is detected only transiently in nascent neurons (Fig. 1F–I). ES organs form via a stereotypic series of asymmetric divisions and each cell, precursor, or terminally differentiated cell, can be distinguished by a unique set of protein markers (Orgogozo et al., 2001). Two ES organ precursors, the 2B and 3B, give rise, respectively, to the multidendritic (MD) and ES neuron. However, neither gives rise to neurons exclusively. Nerfin-1 is transiently expressed transiently in both MD and ES neurons but not in their precursors or in any other cell types in the ES organ lineage (Fig. 1G–I).

Although approximately a third of all early delaminating *nerfin-1* mRNA-positive ventral cord NBs give rise to glia, albeit in varying numbers, we were unable to detect Nerfin-1 protein in glia as judged by co-staining with Nerfin-1 and glial specific markers (data not shown; see Table 1 for glial markers). In addition, as described below, we were unable to identify any defects in glial development due to the loss of *nerfin-1* function (Fig. 3E), indicating that *nerfin-1* is most likely only required for neuronal development and/or function. Taken together, our analysis of *nerfin-1* mRNA and protein expression in the developing embryo reveals that while its message is expressed in many neural precursors and in many nascent neurons, its encoded protein is detected only transiently in a subset of young neurons and in those precursor cells that will undergo a single final division to generate neurons.

Table 1
Nervous system expressed genes examined for altered expression in *nerfin-1*^{null} embryos

Gene	In situ probe template and/or antibodies	Function	Cell-type expression	References
Nuclear				
Abnormal chemosensory jumb 6	mAb anti-Acj6 at 1:30	Transcription factor	Neurons	Clyne et al. (1999); DSHB[a]
Antennapedia	RE64079	Transcription factor	Neurons	Schneuwly et al. (1986)
Castor[b]	Rabbit anti-castor at 1:5000	Transcription factor	NBs, GMCs, neurons	Kambadur et al. (1998)
Couch potato	RE30936	mRNA-binding	Neurons	Bellen et al. (1992)
Cut	Rat anti-CUT F2 at 1:2000	Transcription factor	Neurons	Moore et al. (2004)
Distal antenna related	RE72284	Chromatin constituent	Neurons	Emerald et al. (2003)
Embryonic lethal, abnormal vision[b]	mAb Elav-9F8A9 at 1:10	mRNA splicing	Neurons	Robinow et al. (1988), O'Neill et al. (1994) DSHB
Engrailed	mAb 4D9 Anti-engrailed at 1:20	Transcription factor	NBs, GMCs, neurons	Fjose et al. (1985), Patel et al. (1989); DSHB
Even-skipped[b]	Rabbit even-skipped at 1:5000	Transcription factor	Neurons	Macdonald et al. (1986), Frasch and Levine (1987)
Fruitless	Anti-Fru^{COM} at 1:500	Transcription factor	NBs, GMCs, neurons, glia	Lee et al. (2000), Song et al. (2002)
Nk6 (HGTX)	Rat anti-Nk6.1 at 1:1000	Transcription factor	NBs, GMCs, neurons	Unpublished; gift from J. Skeath
Hunchback[b]	Rat anti-hunchback at 1:10000	Transcription factor	NBs, GMCs, neurons	Tautz et al. (1987); gift from P. Macdonald
Kruppel	RE30918	Transcription factor	NBs, GMCs, neurons	Rosenberg et al. (1986)
Longitudinals lacking	RE13371	Transcription factor	NBs, GMCs, neurons	Crowner et al. (2002)
Nerfin-1[b]	g. pig anti-Nerfin-1 1:5000	Transcription factor	NBs, GMCs, neurons	This publication
Nubbin (Pdm-1)	Rabbit anti-Pdm-1 1:2500	Transcription factor	NBs, GMCs, neurons	Cockerill et al. (1993)
Odd-skipped[b]	Anti-Odd-skipped at 1:3000	Transcription factor	NBs, neurons	Ward and Coulter (2000)
Olig family	GH17679	Transcription factor	GMCs	Brody et al. (2002)
Prospero[b]	mAb MR1A anti-prospero at 1:4	Transcription factor	NBs, GMCs, neurons	Vaessin et al. (1991); DSHB Campbell et al. (1994)
Reversed polarity[b]	mAb 8D12 anti-repo 1:10	Transcription factor	Glia	Xiong et al. (1994)
Target of Pox-n (tap)	RE52048	Transcription factor	Neurons	Bush et al. (1996)
Tyrosine kinase-related protein	GH08821	Chromatin constituent	Neurons	Haller et al. (1987)
Cytosolic				
Bifocal	RE32484	Cytoskeleton	Neurons	Bahri et al. (1997)
Cyclin-dependent kinase 5	RH12210	Signaling	Ubiquitous, neurons	Sauer et al. (1996)
Commissureless[b]	RE60050; rabbit anti-commissureless at 1:50	Protein degradation	Neurons	Tear et al. (1996); DSHB Georgiou and Tear (2002)
Dreadlocks	RE18591	Signaling	Neurons	Garrity et al. (1996)
Futsch[b]	mAb 22C10 at 1:800	Cytoskeleton	Neurons (axons)	Hummel et al. (2000) DSHB
G protein oα47A	RE31907	Signaling	Neurons	de Sousa et al. (1989)

Table 1 (*continued*)

Gene	In situ probe template and/or antibodies	Function	Cell-type expression	References
Cytosolic				
G protein sα60A	RE73364	Signaling	Neurons	Quan et al. (1989)
Highwire	mAb 6H4 at 1:70	E3 ligase	GMCs	Wan et al. (2000); DSHB
Lin-28	RE05342	mRNA binding	NBs, GMCs	Moss and Tang (2003)
PAK-kinase	RE43055	Signaling	Neurons	Harden et al. (1996)
Pod1[b]	g. pig anti-Pod1 at 1:2000	Cytoskeleton	Neurons	Rothenberg et al. (2003)
Smooth	GH05823	mRNA binding	Neurons	zur Lage et al. (1997)
Rhea	TB005630	Cytoskeleton	Neurons	Brody et al. (2002)
Trio	Genomic DNA	Signaling	Neurons	Liebl et al. (2000)
Membrane/extracellular				
Capricious	RE72893	Receptor	Neurons	Taniguchi et al. (2000)
CadherinN	RH17903	Receptor	Neurons	Iwai et al. (1997)
CG13920	RH35836	Putative TM	Neurons	Brody et al. (2002)
Connectin	RE06580	Receptor	Neurons	Nose et al. (1992)
Derailed	RE45978	Receptor kinase	Neurons	Callahan et al. (1995)
Eph receptor tyrosine kinase	RE61046	Receptor	Neurons	Scully et al. (1999)
Ephrin	RE65018	Receptor	Neurons	Bossing and Brand (2002)
Fasciclin 1	RH03873	Receptor	Neurons	Zinn et al. (1988)
Fasciclin 2	RE04334; mAb 1D4 at 1:20	Receptor	Neurons	Grenningloh et al. (1991), Vactor et al. (1993); DSHB
Frazzled	Genomic DNA; rabbit anti-Frazzled at 1:2000	Receptor	Epithelia and neurons	Kolodziej et al. (1996)
Leukocyte-antigen-related-like	GH14613	Receptor phosphatase	Neurons	Krueger et al. (1996)
Late bloomer	mAb 10C9 anti-late bloomer at 1:200	Transmembrane	Neurons	Kopczynski et al. (1996); DSHB
Leak (robo2)[b]	RE46062; rabbit anti-Robo2 at 1:2000	Receptor	Neurons	Rajagopalan et al. (2000)
Netrin A	RE11206	Ligand	Glia	Mitchell et al. (1996), Harris et al. (1996)
Netrin B	GH10173	Ligand	Glia	Mitchell et al. (1996), Harris et al. (1996)
Neuroglian	mAb BP104 at 1:20	Receptor	Neurons	Hortsch et al. (1990) DSHB
Neurotactin	LD22004; mAb BP106 at 1:30	Receptor	NBs, GMCs, neurons	Hortsch et al. (1990) DSHB Speicher et al. (1998)
Protein tyrosine phosphatase 10D	mAb 45E10 at 1:20	Receptor	Neurons (axons)	Tian et al. (1991)
Protein tyrosine phosphatase 69D	RE06719; mAb 3F11 at 1:50	Receptor	Neurons (axons)	Desai et al. (1994); DSHB
Protein tyrosine phosphatase 99A	GH24886; mAb 3A6 anti-Ptp99D at 1:15	Receptor	Neurons (axons)	Tian et al. (1991); DSHB
Robo3[b]	mAb 15H2 anti-roundabout3 at 1:25	Receptor	Neurons (axons)	Simpson et al. (2000a,b)
Roundabout[b] (robo)	mAb 13C9 anti-Robo 1:60	Receptor	Neurons (axons)	Kidd et al. (1999)
Semaphorin-1	RE36155	Ligand	Neurons	Yu et al. (1998)
Slit	GH09454; mAb C555.6D at 1:200	Ligand	Glia	Rothberg et al. (1990)
Syntaxin-1A	mAb 8C3 at 1:15	Vesicular docking	Neurons	Fujita et al. (1982); DSHB

(continued on next page)

Table 1 (*continued*)

Gene	In situ probe template and/or antibodies	Function	Cell-type expression	References
Membrane/extracellular				
unc-5	AT10053	Receptor	Neurons	Keleman and Dickson (2001)
Wnt oncogene analog 5	Rabbit anti-wnt-5 at 1:150	Ligand	Neurons	Fradkin et al. (2004)
Wrapper	RE42803; mAb 10D3 anti-wrapper at 1:200	Receptor	Glia	Noordermeer et al. (1998) DSHB
BP 102[b]	mAb BP 102 at 1:500	Unknown	Neurons (axons)	Klambt et al. (1991) DSHB

[a] DSHB (Developmental Studies Hybridoma Bank).
[b] Expression also examined in elav-Gal4/UAS-nerfin-1 embryos.

Isolation and characterization of nerfin-1 null mutations

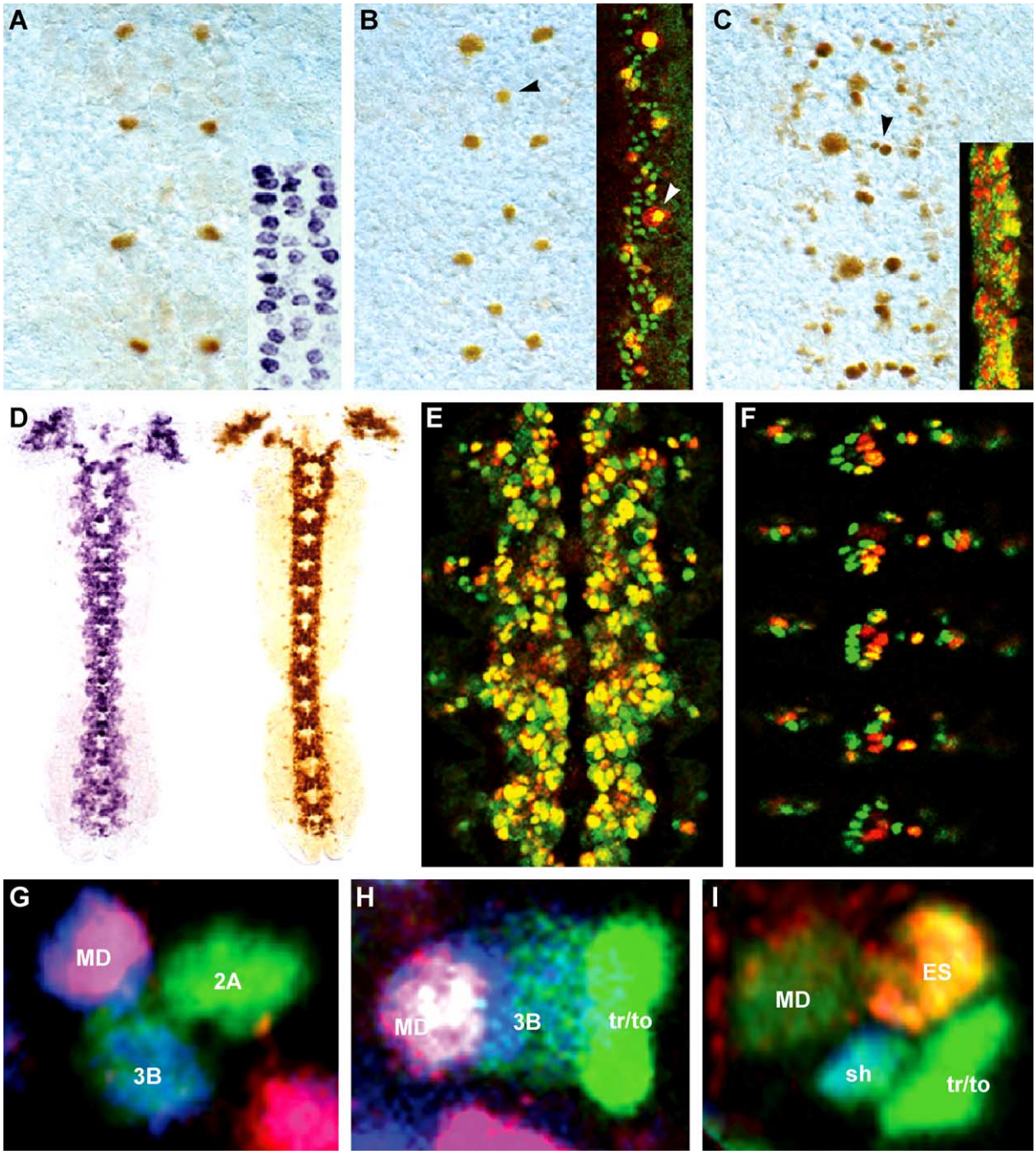
Loss-of-function *nerfin-1* mutations were generated by the "ends-in" homologous recombination gene knockout technique (Rong and Golic, 2001). DNA sequence analysis of the targeted *nerfin-1* locus, after the initial "ends-in" homologous recombination event, revealed that one of the tandem copies of *nerfin-1* had suffered a 593-bp deletion in the transcribed region [Fig. 2A, see *Df(3L)nerfin-1*^{54} breakpoints]. This deletion was most likely caused by exonuclease digestion of the targeting vector after the Sce1 endonuclease-induced double-stranded break but before its integration into the *nerfin-1* chromosomal locus. Deletions covering the minimal promoter and 5′ transcribed leader sequence of both the *Df(3L)nerfin-1*^{54} and *Df(3L)nerfin-1*^{159} alleles (hereafter referred to as *nerfin-1*^{null} alleles) were detected after the allelic substitution step and were most likely the result of illegitimate recombination between micro-homologies present in the minimal promoter of one copy of the *nerfin-1* duplication and the transcribed region of the tandem copy (Fig. 2A). Using conventional X-ray and di-epoxybutane mutagenesis procedures, additional *nerfin-1* mutant alleles were generated from the *mini-white* gene tagged *nerfin-1* locus obtained from the first phase of the knockout targeting technique. Genomic DNA PCR analysis of these larger deletions revealed that both the proximal promoter region and transcribed sequence of *nerfin-1* were removed (Fig. 2A).

The targeted gene knockout and classical mutagenesis screens resulted in the isolation of five independent embryonic recessive lethal alleles. Although late-stage homozygous mutant embryos appeared normal, with no detectable gross morphological or segmentation defects, they failed to hatch from their egg chambers (data not shown). Whole-mount Nerfin-1 immunostaining using both N- and C-terminal directed antisera and mRNA localization revealed that all of the alleles were embryonic nulls for *nerfin-1* expression (Fig. 2C; and data not shown). To confirm that the lethality and cellular phenotype observed in the mutant embryos (see below) was due to the loss of *nerfin-1*, two independent 2nd chromosome P-element insertions that contained an 11,154 bp *nerfin-1* genomic DNA fragment (see Supplemental materials) were used to rescue the viability and cellular phenotype and to restore the *nerfin-1* wild-type expression level (Figs. 2B and D; Fig. 3F).

Fig. 1. Nerfin-1 expression during embryonic development. Dissected tissue fillets of whole-mount immunostained or in situ hybridized embryos are shown. (A–C) Stages 9, 10, and 11, respectively, showing the ventral cord 3rd thoracic and abdominal 1–3 segments. (A) Nerfin-1 protein is first detected in ventral cord MP2 NBs. Inset; at this time, most, if not all, early delaminating NBs express *nerfin-1* mRNA (inset shows mRNA localization of the right half of the 3rd thoracic and 1–3 abdominal segments). (B) Soon after the appearance of Nerfin-1 protein in the MP2, Nerfin-1 is next observed in midline MP1 NBs (black arrowhead). Inset in B window: White arrowhead highlights the nuclear co-localization of Nerfin-1 (red) and Pros (green) in the MP2. Shown is a tilted, dorsolateral view (~45°) of abdominal segments 1–4 (oriented so that dorsal is left). (C) Nerfin-1 is next detected in the midline MP3 NB and in the MP2 daughter cell nuclei, the vMP, and dMP interneurons (arrowhead). During stage 11, Nerfin-1 is also expressed in a subset of GMCs. Inset in C window: Double-labeling of *nerfin-1* mRNA (green) and Pros protein (red) reveals that *nerfin-1* mRNA is expressed in most, if not all, GMCs [shown is a tilted, dorsolateral view (~45°) of thoracic and abdominal segments, oriented so that dorsal is left]. (D) By stage 12, many GMCs and nascent neurons throughout the developing CNS have detectable levels of *nerfin-1* mRNA (left) and protein (right). (E, F) Stage 13, double-labeling for Nerfin-1 (red) and Pros (green) proteins reveals that many, but not all, Pros expressing ventral cord GMCs and nascent neurons also express Nerfin-1 (E). In the developing PNS (F), Nerfin-1/Pros co-labeling reveals that Nerfin-1 is expressed in nascent neurons (shown are abdominal external sensory and cordotonal organs). (G–I) An elaborating abdominal ventral pore external sensory organ labeled with antibodies to detect Cut (green), Pros (blue), and Nerfin-1 (red). Cut labels all cells of the elaborating organ. In the 1 and 2 cell stages of organ elaboration, there is no expression of Nerfin-1 (not shown). (G) Nerfin-1 is first seen in the 3-cell stage. This stage consists of two intermediate cells the 2A and 3B and the 3B-sibling multidendritic (MD) neuron. The 3B and the MD neuron both express Pros, in addition the MD neuron expresses Nerfin-1. (H) In the 4-cell stage organ, the 2A precursor has divided to form the non-neural trichogen (tr) and tormagen (to) cells; furthermore, in this panel, the 3B cell is undergoing division. (I) In the 5-cell organ, The 3B has divided to give the ES neuron and glial/sheath cell. Pros expression in the MD and ES neurons is transient but remains on in the glial/sheath (sh) cell. Nerfin-1 is now present in the ES neuron. However, Nerfin-1 expression has been lost from the MD neuron, illustrating its transient nature.

Loss of nerfin-1 does not significantly alter nervous system lineage development

To determine if neural lineage development or the expression of known neural precursor, neural-, or glial-identity genes were altered in *nerfin-1*^{null} embryos, we next examined the expression of 19 genes that have been demonstrated to play important roles in these early events (see Table 1 for a list of genes examined). In summary, the analysis of all tested cell-identity markers revealed that the developmental processes that give rise to the correct numbers and identities of neurons and glia in both the CNS and PNS were not significantly affected by the loss of *nerfin-1* function. For example, the spatial and temporal expression dynamics of Elav (neuronal) and Wrapper/Slit/Repo (glial) identity markers were not altered in *nerfin-1*^{null} embryos (Fig. 3 and data not shown). In addition, expression of the transcription factors Hunchback, Kruppel, Pdm-1, Castor, Pros, Engrailed, Eve, and Odd-skipped were indistinguishable between wild-type and mutant embryos (data not shown). Although we cannot rule out the possibility that more subtle changes in neuronal identities have occurred as a result of loss of *nerfin-1*, our analysis indicates that neurons and glia have not suffered major changes in their identities.



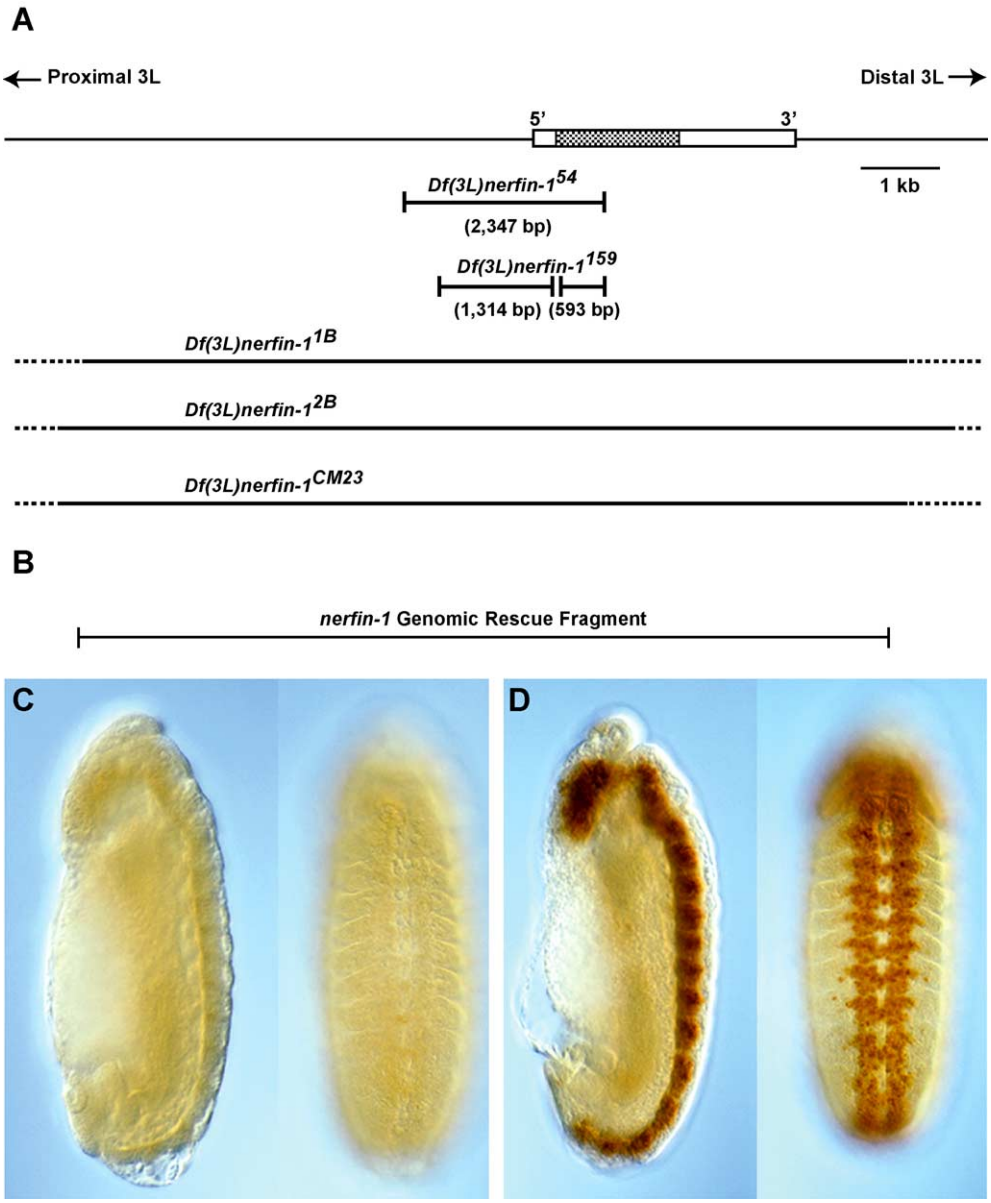


Fig. 2. *nerfin-1* genomic locus, deficiencies, and genomic rescue. (A) Cartoon represents 13 kb of genomic DNA that spans the intronless *nerfin-1* gene, located on the 3rd chromosome at cytological location 61D4. The rectangular box denotes the 5′ and 3′ boundaries of the longest identified transcribed region (3205 bp) deduced from the cDNA sequence of clones RE67852 and LD18634 (obtained from BDGP). The stippled region within the transcribed sequence represents the transcript's longest ORF (1407 bp). Chromosomal breakpoints and deleted regions of deficiencies *Df(3L)nerfin-1*^{54} and *Df(3L)nerfin-1*^{159}, generated by the homologous recombination gene knockout technique (marked with thick black bars), are aligned below the genomic map and the size (bp) of each deletion is shown in parenthesis. In addition, two independent X-ray-induced deficiencies (*Df(3L)nerfin-1*$^{1B\ and\ 2B}$) and one di-epoxybutane triggered deficiency (*Df(3L)nerfin-1*^{CM23}) are delineated with thick black bars with breakpoint uncertainties highlighted with dotted lines. (B) Aligned with the cartoon is an 11,095-bp genomic DNA rescue fragment that contains the transcribed region plus 5786 and 2130 bp of flanking 5′ and 3′ DNA, respectively. (C) Nerfin-1 immunostaining using different polyclonal antisera failed to detect any significant immunoreactivity in embryos homozygous for the *Df(3L)nerfin-1*^{54} deficiency [shown are lateral (left) and ventral (right) views of a late stage 12 embryo]. (D) Nerfin-1 immunoreactivity is restored to apparent wild-type levels in *Df(3L)nerfin-1*^{54} homozygous embryos that are also homozygous for a 2nd chromosome P insertion carrying the genomic DNA rescue fragment (same stage and orientations as in C).

nerfin-1 is required for CNS axon patterning

Given the absence of any detectable alteration in NB-lineage development in *nerfin-1*^{null} embryos, we next sought to determine if Nerfin-1 played a more restricted role in neuronal maturation, such as axon outgrowth and/or pathfinding. To assess if axon patterning was altered in *nerfin-1*^{null} embryos, a battery of antibody markers was used to identify many axons or to decorate specific subsets of axons in the CNS and PNS (Figs. 3 and 4; see Table 1 for antibodies used).

Immunostains of *nerfin-1*^{null} embryos revealed significant alterations in axon projections within the embryonic CNS but not in the PNS. For example, within the ventral

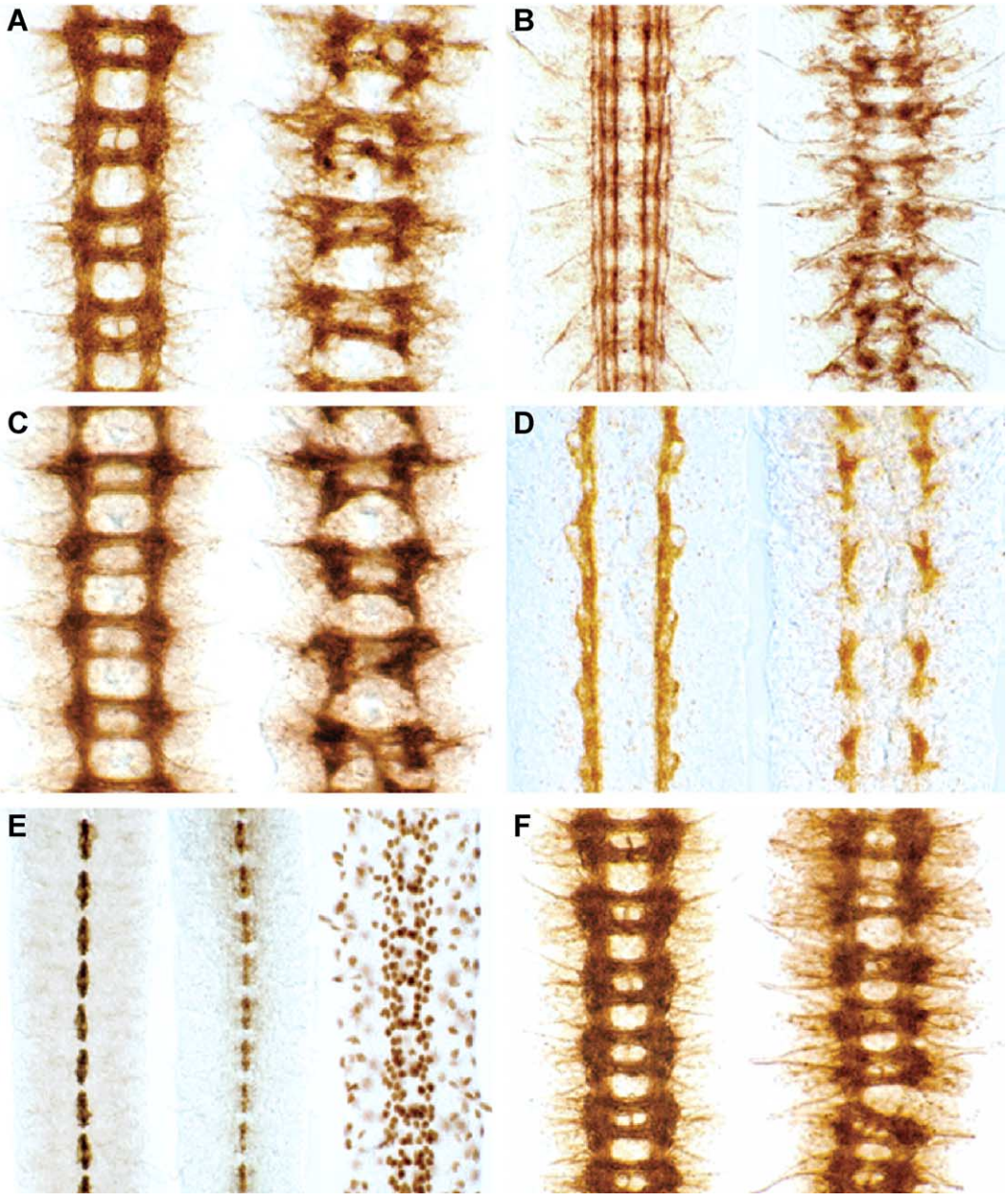
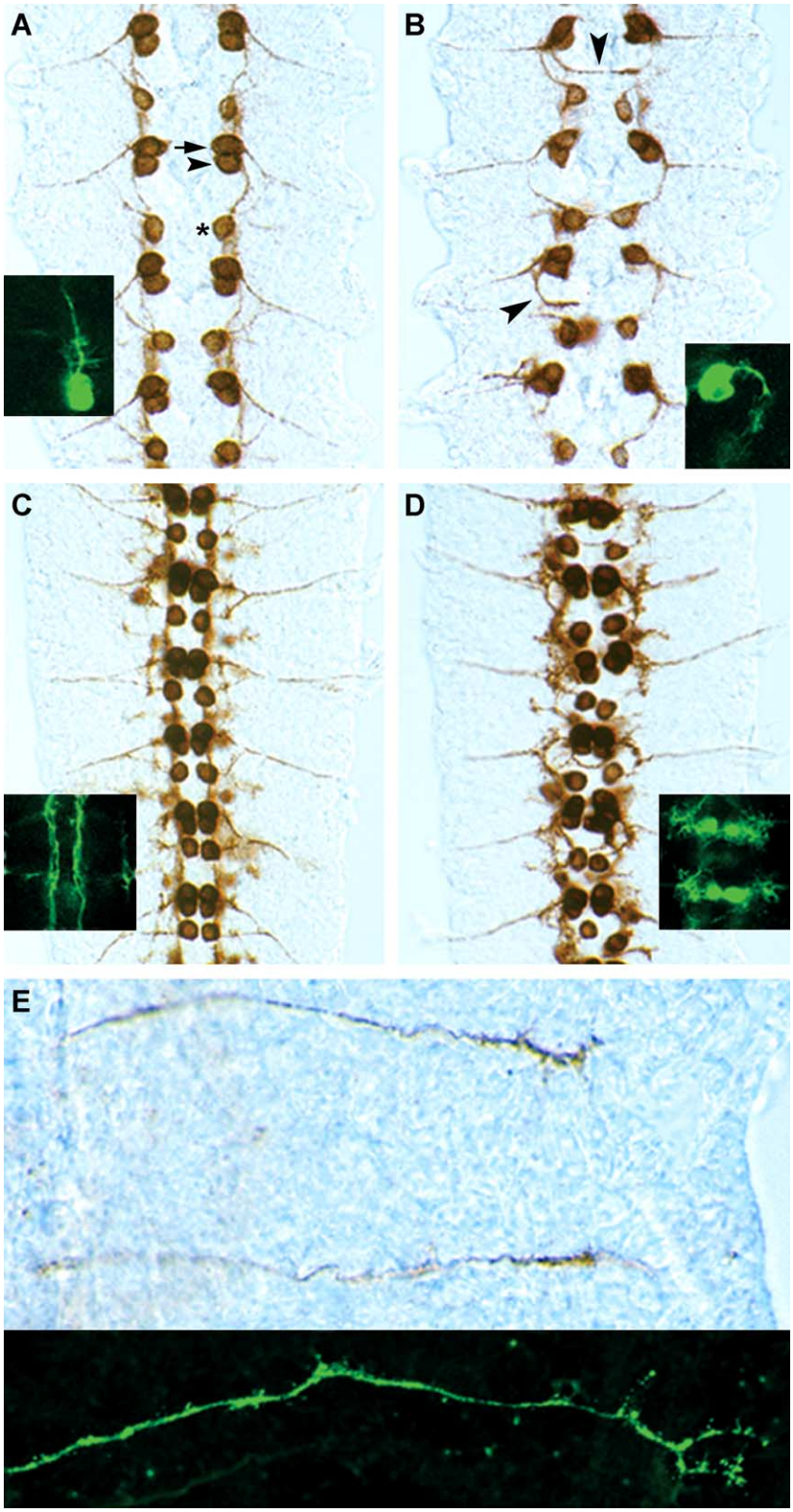


Fig. 3. Nerfin-1 is required for correct CNS axon fascicle patterning. (A–D) Each panel shows a wild-type (left) and *Df(3L)nerfin-1*^{54} mutant (right) ventral cord. Dorsal views of ventral cords dissected from embryos immunostained with different axon markers: BP102 antibody (A, stage 14; F, stage 15), anti-Fas2 (B, stage 16), anti-Ptp10D (C, stage 14), anti-Robo-3 (D, stage 14); or glial markers (E, stage 14) anti-Wrapper (left), anti-Slit (center), and anti-Repo (right). In *Df(3L)nerfin-1*^{54} mutant embryos, most axons within longitudinal connectives fail to project across segmental boundaries and frequently terminate in tangles at or near the boundaries. In addition, both the anterior and posterior commissures of each segment fail to development properly (see panels A–D). (B) By stage 15, Fas-II-positive longitudinal tracks are significantly disorganized. (D) Robo3-positive axons also fail to project to adjacent segments. (E) Wild-type expression levels of the glial-specific proteins Wrapper, Slit, and Repo are observed in *Df(3L)nerfin-1*^{54} embryos. (F) BP102 immunostaining reveals that the axon patterning defects observed in the *Df(3L)nerfin-1*^{54} mutant embryos can be rescued by second chromosomes carrying a *nerfin-1* genomic DNA rescue fragment (right); however, only partial rescue was obtained with *Elav.Gal4/UAS.nerfin-1* transgenes (left).

nerve cord of stage 13 and older *nerfin-1*^{null} embryos, the longitudinal connective axon fascicles were disrupted between segments, and both the anterior and posterior commissures of each ventral cord ganglia were malformed (Fig. 3; 100% penetrant, >55 embryos examined). Axons that normally project through fascicles that make up the intersegmental longitudinal connectives appeared to either stall or randomly turn at or near segmental boundaries, creating disorganized tangles (Fig. 3). In addition, the organization of longitudinal connectives within each of the segments was abnormal with misrouted axons projecting laterally away from the longitudinal tracks (Fig. 3D). Immunostains also revealed that the overall axon fascicle organization and apparent axon density of the ventral cord

commissures was affected by the loss of *nerfin-1* function (Fig. 3). In addition, BP102 immunostaining of stage 14 and older embryos showed that the diameters of both the supra- and sub-esophageal commissures of the brain were significantly reduced in loss-of-function mutants (data not shown; 100% penetrant, >45 embryos examined). In stage 15 and older mutants, the medial, intermediate, and lateral Fasciclin2 (Fas2) positive longitudinal tracks were disrupted



along the entire length of the ventral cord (Fig. 3B; 100% penetrant, >50 embryos examined).

In contrast to the axon fascicle organization defects observed in the ventral cord and brain, no significant patterning defects were detected in the motoneuron nerve tracts that exit the CNS (Figs. 3 and 5 and data not shown). In addition, the axon patterning of PNS neurons, outside the CNS, also appeared normal in *nerfin-1*^{null} embryos (data not shown).

CNS interneurons require nerfin-1 function for early axon guidance

To better understand the axon misrouting in the CNS of *nerfin-1*^{null} embryos, analysis next focused on the axonal development of three ventral cord neurons, the pCC interneuron, and the aCC and RP2 motoneurons (for details of their development and axon projections, see Schmid et al., 1999). To accomplish this, a CD8GFP expressing *Gal4/UAS* transformant line was employed that prominently marks cell bodies and axons of these neurons (Fujioka et al., 2003). Nerfin-1 and Eve expression transiently overlap in these neurons, as judged by co-nuclear localization; however, Eve expression was not altered by loss of *nerfin-1* (data not shown).

In the absence of *nerfin-1* function, significant patterning defects were observed in the pCC axons, which normally send their pioneering ipsilateral axons anteriorly to establish the medial fascicle of the longitudinal connective tracks (Fig. 4). In stage 13 *nerfin-1*^{null} embryos, the pCC interneurons failed to send their axons anteriorly and instead projected their axons either in a lateral or posterior direction and many of the posterior projecting pCC axons crossed the ventral midline in adjacent posterior segments (Fig. 4B arrowheads). By stage 14, all pCC interneurons in *nerfin-1*^{null} embryos had misguided axons and many of these axons had extensive side branches (Fig. 4D; 100% penetrant, >35 embryos examined). In contrast, when compared to the significant axon misguidance phenotype of the pCC interneurons, the overall development and patterning of the aCC and RP2 motoneuron axon tracts did not appear to be adversely affected in *nerfin-1*^{null} embryos (Fig. 4, panels B–E). For example, in *nerfin-1*^{null} embryos, the aCC and RP2 axons

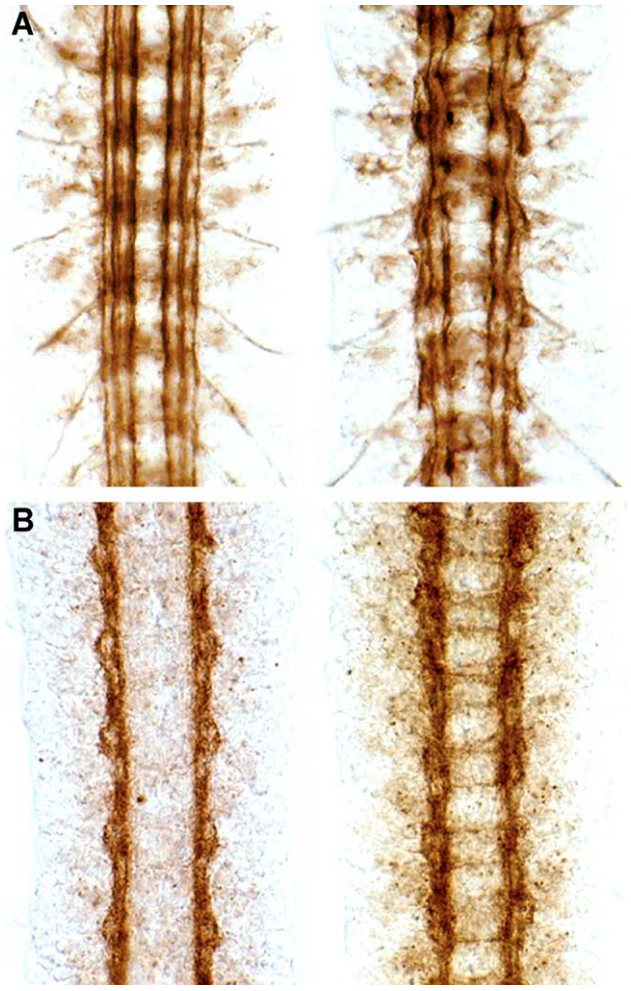


Fig. 5. Prolonged misexpression of Nerfin-1 in neurons triggers defects in longitudinal connective axon fasciculation. Misexpression of Nerfin-1 in neurons via *elav.Gal4/UAS.nerfin-1* transgenes results in defasciculation of longitudinal axon connectives (A) and in the misexpression and/or altered axonal distribution of Robo3 (B). Shown are dorsal views of dissected ventral cores from stage 15 wild-type embryos (left side) and *elav.Gal4/UAS.nerfin-1* embryos (right side). (A) Misexpression of Nerfin-1 results in the defasciculation of Fas2-positive longitudinal axon connectives. Of the three Fas2-positive fascicles, the last to form, the lateral most longitudinal fascicle has the most breaks. (B) Immunostains with anti-Robo3 antibody reveals that neuronal misexpression of Nerfin-1 triggers the ectopic distribution of Robo3 outside of the medial longitudinal axon fascicles. Note the Robo3-positive axons crossing the midline.

Fig. 4. Loss of *nerfin-1* disrupts pCC interneuron axon patterning without significantly altering the initial outgrowth of aCC and RP2 motoneuron axons. In *Df(3L)nerfin-1*^{54} embryos, most, if not all, pCC interneuron axon projections are abnormal. (A) The pCC (arrowhead), aCC (arrow), and RP2 (asterisk) neurons were identified by their expression of membrane associated GFP (see Supplemental materials for details of *eve-Gal4/UAS-CD8GFP* transgenes). Dissected, GFP-immunostained ventral cord fillets (A–D) or flattened embryos (E). Wild-type (A and C) and *Df(3L)nerfin-1*^{54} (B, D, and E) embryos. Panel insets are GFP-fluorescent images from live embryos viewed in whole-mount showing pCC cell bodies and/or axons (A, B, and D), just pCC axons (C) or of an unfixed flattened embryo preparation showing the aCC/RP2 laterally projecting axon track (E). (A, B) Stage 13 ventral cords showing the 3rd thoracic and first three abdominal segments. Instead of normally extending ipsilaterally in an anterior direction into the flanking segments (A), many pCC axons in mutant embryos (B) reverse direction, extend posteriorly, and cross the midline (arrowheads, also compare insets). (C, D) Stage 15 ventral cords showing dorsal views of 2nd and 3rd thoracic and first three abdominal segments. By late stage 14, most pCC axons in mutant embryos display misguidance defects and are highly branched (D and inset). (B, D, and E) aCC and RP2 motoneuron axon projections do not appear to be significantly altered in *nerfin-1* loss-of-function mutants. (E) aCC and RP2 motoneuron axon projections appear to extend to their synaptic targets in the dorsal muscle field in mutant embryos. Shown is one side, 3rd thoracic, and first abdominal segments of a stage 15 flattened embryo (ventral cord is to the right). Inset shows a GFP-positive aCC/RP2 axon tract from an abdominal segment of a *Df(3L)nerfin-1*^{54} embryo.

project to the dorsal muscle field, which contains the synaptic targets of these motoneurons. It should be noted that although *nerfin-1* function does not appear to be required for these motoneurons to project their axons to the appropriate synaptic target field, the role of Nerfin-1 in synaptic target choice has not yet been fully assessed.

Prolonged expression of Nerfin-1 in neurons triggers defects in CNS axon fasciculation

The transient nature of Nerfin-1 expression in nascent neurons suggests its role may be restricted to a specific phase of early axon guidance and that its presence in the nuclei of neurons undergoing subsequent phases of axon guidance and/or maturation may interfere with these processes. To determine the significance of the temporally restricted expression, we studied the effects of Nerfin-1 misexpression outside of its normal wild-type expression boundaries. Targeted misexpression of Nerfin-1 was accomplished by the yeast *Gal4/UAS* system (Brand and Perrimon, 1993; Fischer et al., 1988). Using different *Gal4* driver lines (*scabrous-*, *pros-*, or *castor-Gal4*; see Supplemental materials for description of the *Gal4* drivers) to activate the expression of *UAS*-linked *nerfin-1* during different stages of NB-lineage development, we observed that misexpression did not alter neuronal or glial development nor did it affect axon fascicle patterning. In addition, the ectopic expression of Nerfin-1 in mesodermal-derived tissues, outside of the nervous system, via a *twist-Gal4* driver, had no detectable effect on muscle development or embryonic viability (data not shown).

However, prolonged/extended expression in neurons resulted in embryonic lethality and ventral cord axon fascicle patterning defects. Whole-mount immunostains of late stage 14 and older *elav-Gal4/UAS-nerfin-1* embryos identified multiple defects in axon scaffolding throughout the CNS. Prolonged expression in neurons interfered with the development of Fas2-positive longitudinal connective fascicles (Fig. 5A; 100% penetrant, >40 embryos examined). These experiments also revealed that Nerfin-1 misexpression had a differential effect on the organization of Fas2 positive axon fascicles, with the intermediate and lateral fascicles exhibiting a greater degree of defasciculation than the medial fascicle (Fig. 5A). Immunostains with antibodies specific for the different Robo family members also revealed that misexpression of Nerfin-1 in neurons affected the distribution of Robo3. Robo3 exhibited a wider more diffuse ventral cord distribution in *elav-Gal4/UAS-nerfin-1* embryos, and it was found in axons extending across the midline, suggesting either that the subcellular distribution of Robo3 had been altered or that axons within the Robo3-positive axons had defasciculated from the connectives and crossed the midline (Fig. 5B). Interestingly, no significant effect on the other two Robos, Robo and Robo2, were observed in *elav-Gal4/UAS-nerfin-1* embryos (data not shown). In addition, no significant changes in the expression of other axon guidance genes or cell-fate determinants were detected in *elav-Gal4/UAS-nerfin-1* embryos (see genes in Table 1 denoted with "b"). Prolonged misexpression did not significantly alter the patterning of motoneuron axon tracks that exit the CNS nor did it adversely affect axon patterning in the PNS (Fig. 5A and data not shown).

A screen for potential targets of Nerfin-1 regulation identifies a subset of genes involved in axon guidance

Given the axon guidance defects in *nerfin-1*^{null} embryos and the fact that Nerfin-1 is a Zn-finger nuclear protein, we hypothesized that Nerfin-1 may be required for the correct expression of genes involved in axon guidance. Accordingly, the embryonic expression profiles of over 35 genes that have been shown to play important roles in axon guidance were examined. Included in the candidate screen were genes encoding transcription factors, RNA-binding proteins, cell surface receptor proteins, their ligands, signal transduction proteins, and components of the cytoskeleton (see Table 1 for list of genes). Homozygous *nerfin-1*^{null} embryos were identified by the absence of Nerfin-1 immunoreactivity. Whole-mount in situ hybridization and/or protein immunostaining for altered spatial or temporal expression in *nerfin-1*^{null} embryos identified six genes that require *nerfin-1* function to achieve full wild-type expression levels (Fig. 6).

Two genes involved in anterior vs. posterior commissure choice, those encoding the receptor tyrosine kinase Derailed, and its ligand Wnt5, were both required *nerfin-1* for full expression (Figs. 6A, B). In the absence of *nerfin-1*, ventral cord expression levels of Robo and Robo3 were unaffected; however, Robo2 expression levels were significantly reduced (Fig. 6C). Expression of Slit, the ligand for Robo receptors, and Commissureless, a factor responsible for clearing Robo receptors from commissural axons (reviewed by Schnorrer and Dickson, 2004), were unaffected in *nerfin-1*^{null} embryos (data not shown).

Loss of *nerfin-1* function also significantly delayed and/or reduced the early expression of the neuron-specific microtubule-associated MAP1B-like gene *futsch*. *futsch* expression is normally activated in newborn neurons starting at stage 11; however, in *nerfin-1*^{null} embryos expression is first detected only at the stage 13 (Fig. 6D). Not until embryonic stage 15 did the level of *futsch* expression in mutant embryos approach that of wild type (data not shown). Reduced mRNA steady state levels for the genes encoding Leukocyte-antigen-related-like (Lar; Krueger et al., 1996), another receptor tyrosine kinase, and *G-oα47A* gene (Yoon et al., 1989), which encodes an alpha subunit of heterotrimeric G proteins, were also detected in *nerfin-1*^{null} embryos (Figs. 6E, F). The reduced level of gene expression in mutant embryos was nervous system specific. For example, *G-oα47A* gene expression in mesodermal derived tissues was not altered in *nerfin-1*^{null} embryos (Fig. 6F).

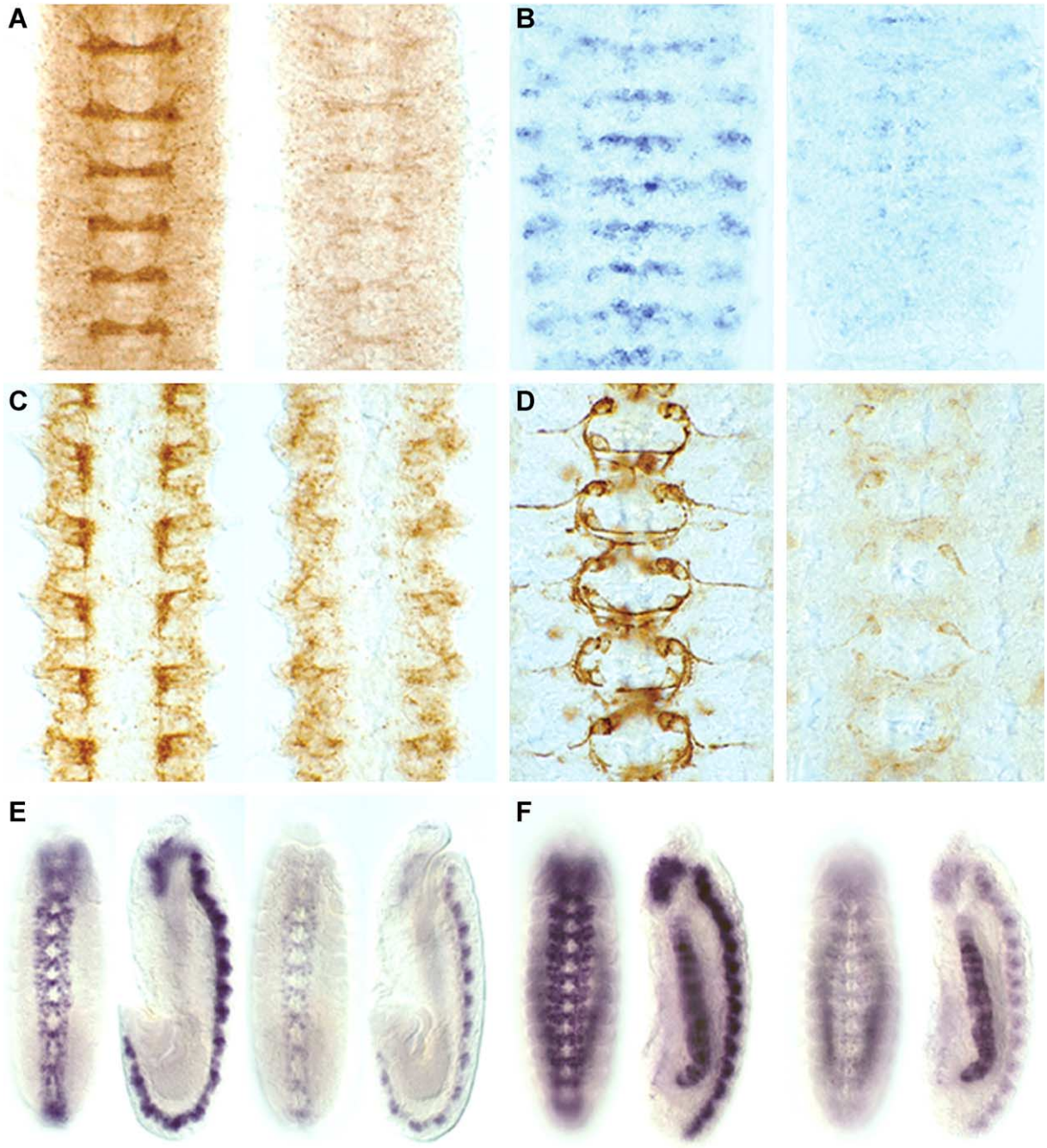


Fig. 6. *nerfin-1* is required for the wild-type expression levels of a subset of genes involved in axon pathfinding. In each panel, wild-type is left and *Df(3L)nerfin-1*^{54} ventral cords or embryos are right. Immunostains (A, C, and D) or in situ hybridizations (B, E, and F) are shown. *nerfin-1* is required for wild-type expression levels of Wnt5 (A, stage 14), *derailed* (B, stage 15), Robo2 (C, late stage 13), Futsch (D, early stage 13), *dlar* (E, stage 12/3), and *G-oα47A* (F, stage 13). Shown are dorsal views of ventral cords fillets (A–D) and whole-mount embryo (E,F) ventral and lateral views.

nerfin-1 requires pros but not lola or fru for wild-type expression

Comparative analysis of Nerfin-1 and Pros expression in the developing nervous system revealed a marked, although not complete, overlap in expression. The nuclear co-localization of these proteins in neuronal precursor cells, and the fact that mutations in *nerfin-1* (this report) and *pros* (Doe et al., 1991; Matsuzaki et al., 1992; Vaessin et al., 1991) trigger axon guidance defects, raised the possibility that they may regulate the expression of one another. To determine the epistatic relationship between *nerfin-1* and *pros*, we studied their expression dynamics in each other's loss-of-function mutant backgrounds. Pros immunostaining in *nerfin-1*^{null} embryos did not identify any significant changes in Pros expression (data not shown). In marked contrast,

nerfin-1 mRNA and protein levels in embryos collected from two independent loss-of-function *pros* mutants revealed that *pros* is required for wild-type *nerfin-1* expression levels (Figs. 7A and B and data not shown). Nerfin-1 expression was significantly reduced throughout the CNS and PNS; however, its expression was not completely ablated in any of the $pros^{null}$ alleles tested (see Supplemental materials for *pros* alleles).

To determine if the axon guidance phenotype observed in $pros^{null}$ ($pros^{I13}$; Bi et al., 2003) mutant embryos could be explained by a requirement for wild-type *nerfin-1* expression, the extent of CNS axon disorganization triggered by single and double mutant combinations were examined. Comparisons demonstrated that loss of *pros* function resulted in a more severe phenotype than that observed in the $nerfin\text{-}1^{null}$ embryos (Figs. 7D, E). Although both $pros^{null}$ and $nerfin\text{-}1^{null}$ embryos exhibited disruptions in the longitudinal axon connectives, loss of *pros* function resulted in an overall greater disruption in commissure organization. In addition, the nerve cord in $pros^{null}$ embryos was considerably wider when compared to *nerfin-1* mutants or wild-type embryos (Fig. 7; also see Discussion). The axon scaffolding phenotype observed in $nerfin\text{-}1^{null}$; $pros^{I13}$ double mutant was more severe than that of either single mutant (Fig. 7F).

Analysis of two other transcription factor genes that are widely expressed in the developing CNS, *lola* and *fru*, has revealed that they too are required for proper axon guidance and both are required for proper longitudinal axon fasciculation (Giniger et al., 1994; Song et al., 2002). However, unlike the severe axon guidance phenotype observed in $pros^{null}$ embryos, the disorganization of the

Fig. 7. *pros* is required for the proper Nerfin-1 expression. Nerfin-1 expression is significantly reduced in $pros^{I13}$ mutant embryos. (A, B) Shown are whole-mount Nerfin-1 immunostained stage 13 wild-type (A, dorsal and ventral views) and $pros^{I13}$ homozygous embryos (B, same views as in A). (C–F) $Df(3L)nerfin\text{-}1^{54}/pros^{I13}$ double mutants have a more severe axon guidance phenotype than either $Df(3L)nerfin\text{-}1^{54}$ or $pros^{I13}$ null alleles. Ventral views of whole-mount embryos stained with BP102 to reveal the axon scaffold (C) wild-type, (D) $Df(3L)nerfin\text{-}1^{54}$, (E) $pros^{I13}$, and (F) $Df(3L)nerfin\text{-}1^{54}$; $pros^{I13}$ double mutant. Both $nerfin\text{-}1^{null}$ and $pros^{I13}$ mutations disrupt longitudinal connectives development; however, the $pros^{I13}$ phenotype is more severe and the double mutant phenotype is more severe than either single mutant. Note the wider ventral cord in the $pros^{I13}$ mutants (see Discussion).

CNS axon scaffolding is not as extensive in *lola* or *fru* loss-of-function mutants. To determine the epistatic relationship between *nerfin-1* and *lola* or *fru*, the expression of each gene in each other's loss-of-function background was analyzed. In contrast to the marked reduction of *nerfin-1* expression in *pros* mutants, no such reduction in *nerfin-1* expression was found in *lola* or *fru* mutants, nor was the expression of *lola* or *fru* altered in *nerfin-1*^{null} embryos (data not shown; see Supplemental materials for description of *lola* and *fru* alleles).

Discussion

The principal finding of this study is that *nerfin-1* is required by many, but not all, CNS neurons for early axon pathfinding. Expression screens of known axon guidance determinants have revealed that a subset of genes involved in different aspects of early pathfinding and fasciculation require *nerfin-1* for full expression.

Nerfin-1 protein expression is tightly regulated during nervous system development

Although *nerfin-1* mRNA expression is first detected in all early delaminating NBs, its encoded protein is detected only in NBs, specifically the MPs, which divide just once to produce interneurons. The transient expression of *nerfin-1* mRNA in NBs, during the early phases of lineage development but not during intermediate or late stages, suggests that its NB expression is subject to temporal regulation (reviewed by Brody and Odenwald, 2002). Following this initial phase of expression, *nerfin-1* mRNA is detected in most if not all GMCs and nascent neurons and, again, Nerfin-1 protein is detected only transiently in a subset of these cells. Nerfin-1 protein is also transiently expressed in nascent PNS neurons. The transient expression of Nerfin-1 in neurons during the initial phases of axon development is consistent with the idea that it may be required for specific aspects of early axon guidance, particularly in CNS interneurons. In addition, the absence of detectable Nerfin-1 protein in many mRNA expressing cells suggests that its message may be translationally blocked and/or that the protein is rapidly degraded in these cells. A recent genome-wide screen for genes whose transcripts contain potential binding sites for the translational blocking micro-RNAs (miRNAs) has revealed that the 3′ UTR of the *nerfin-1* message (1600 bases long) contains multiple predicted docking sites for nine different miRNAs (Enright et al., 2003). Protein instability motifs (PEST sites) within Nerfin-1 may also play a role in rapidly clearing Nerfin-1 from cells.

Pros regulation of nerfin-1 expression

Although no cross-regulation was detected between *nerfin-1* and *lola* or *fru*, *pros* is required for full *nerfin-1* expression. In addition to its role in axon guidance, *pros* has been shown to be required to bring precursor cells out of the proliferative state as a prelude to neuronal proliferative quiescence. Absence of *pros* results in an additional division of the GMC that results in increased numbers of cells in the CNS (Li and Vaessin, 2000). In *nerfin-1*^{null} embryos, we found no evidence to indicate that loss of *nerfin-1* affects precursor or neuron cell numbers nor does it trigger an increase in cell death, suggesting that *nerfin-1* carries out a restricted repertoire of the *pros* functions, specifically those involved with axon guidance.

Nerfin-1 is required for early axon pathfinding

Our analysis of *nerfin-1*^{null} embryos indicates that Nerfin-1 is needed by many, but not all, CNS neurons during the early phases of axon patterning. For example, while the development and patterning of ventral cord longitudinal connective fascicles is disrupted and both AC and PC development is compromised, we were unable to detect any significant disruptions in motoneuron nerve tracks that exit the CNS, nor did we observe significant disruptions in the PNS axon patterning.

It is likely that many of the subsequent defects in axon guidance, related to crossing segmental boundaries, defasciculation, and/or commissural development may have their origins in defects in the initial guidance decisions made by pioneering axons. Disruption in the initial pathfinding events can trigger subsequent misguidance of "follower neurons" that rely on guidance cues laid down by pioneers (Hidalgo and Brand, 1997). The pCC axon misguidance in *nerfin-1* mutants could explain, in part, why many other axons fail to extend across the segmental border in *nerfin-1* mutants. Two aspects of pCC axon guidance are altered in *nerfin-1* mutants. First, the pCC interneuron fails to extend its axon in the proper anterior orientation and, second, many of the misguided axons cross the midline in adjacent posterior segments (Fig. 5B). During normal development, the pCC ipsilateral axon pioneers the innermost longitudinal fascicle, the medial fascicle (Schmid et al., 1999), and other axons project along the tract established by the pCC. Although defects in the initial anterior/posterior direction of pCC axon projection have not been reported in other mutant backgrounds, abnormal crossing of the midline has been observed in *eve* mutants (Fujioka et al., 2003) and also observed in *robo* mutant embryos; axons that normally pioneer ipsilateral projections project anteriorly and then cross the midline, and contralaterally projecting axons re-cross the midline multiple times (Kidd et al., 1998a,b; Seeger et al., 1993). At this time, we do not know whether the follower axons also are defective in navigation, or whether their misguidance is solely due to initial axon pathfinding mistakes made by the pioneering neurons. Given the large numbers of neurons that express Nerfin-1, a scenario that includes both possibilities is favored.

Altered expression of axon guidance determinants in nerfin-1 mutants

Analysis of the expression dynamics of known axon guidance genes in *nerfin-1*^{null} embryos revealed that *nerfin-1* function is required for the proper expression of a subset of factors involved in at least two signaling events essential for early CNS axon patterning. Although Netrin/Frazzled signaling appears not to be affected by loss of *nerfin-1*, we observed that *nerfin-1* function is required for the proper expression of specific genes involved in the decision to cross the midline and in the choice of commissures. One of the components of the Slit/Robo system, Robo2, requires Nerfin-1, either directly or indirectly, for full expression. Interestingly, we did not detect altered expression of the other factors involved in the Slit/Robo pathway, specifically *slit*, *comm*, *comm3*, *robo*, and *robo3* appear unaffected in *nerfin-1*^{null} embryos. Robo2 expression is restricted to axons that extend in the outer longitudinal pathway (the lateral fascicle), farthest from the midline and thus farthest from the source of Slit (Rajagopalan et al., 2000; Simpson et al., 2000b). The *robo2* loss-of-function phenotype does not resemble that of the *nerfin-1*^{null}, suggesting that only a part of the *nerfin-1* loss-of-function phenotype can be derived from its regulation of *robo2*.

nerfin-1 function is also required for the proper expression of Drl and Wnt5, two factors involved in the choice between entering the AC or PC. Both *derailed* and *Wnt5* mutants display abnormal projections of AC axons into the PC (Bonkowsky et al., 1999; Yoshikawa et al., 2003). Although not as severe as in *nerfin-1*^{null} embryos, the loss of *Wnt5* also triggers breaks in longitudinal connectives, specifically the intermediate and lateral fascicles (Fradkin et al., 2004).

Delayed onset of *futsch* expression was observed in *nerfin-1*^{null} embryos. In loss-of-function *futsch* mutant alleles, the development of the lateral-most longitudinal connectives is reduced (Hummel et al., 2000). Some of the axon fasciculation defects seen in *nerfin-1*^{null} embryos could be explained in part by delayed *futsch* expression, especially those exhibited by pioneering axons that make up the lateral fascicle.

Reduced expression of *G-oα47A* and *Lar* were also observed. *G-oα47A* is required for the proper development of Fas2-positive connectives (Fremion et al., 1999). However, the axon guidance phenotype of *G-oα47A* mutants is more severe than in *nerfin-1*^{null} *embryos*; in the *G-oα47A* mutant, AC and PC do not separate. The greater severity in the *G-oα47A* mutant could be explained by the fact that loss of *nerfin-1* does not completely ablate *G-oα47A* expression. *Lar* has been found to regulate motoneuron axon guidance decisions outside of the CNS (Krueger et al., 1996). The lack of motoneuron axon patterning defects in *nerfin-1*^{null} *embryos* could be explained by the fact that *Lar* expression, in mutants, was just reduced and not ablated.

Although some of the axon guidance defects in the *nerfin-1*^{null} mutants can be explained, in part, as a result of the altered expression of the above genes, certain aspects of the patterning defects are distinct from those described for these genes. For example, patterning defects in pCC axons have been observed due to loss of *eve* or *robo*, but the reversed anterior/posterior orientation of many of the pioneering pCC axons in *nerfin-1*^{null} embryos has not been seen in other mutant backgrounds. This suggests that Nerfin-1 may regulate additional, as yet uncharacterized pathfinding determinants. Therefore, it will be very interesting to identify additional genes regulated by Nerfin-1 as these are very likely to include new members of the axon guidance machinery.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2004.09.027.

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